Chronic Therapy With Elamipretide (MTP-131), a Novel Mitochondria-Targeting Peptide, Improves Left Ventricular and Mitochondrial Function in Dogs With Advanced Heart Failure

Hani N. Sabbah, PhD; Ramesh C. Gupta, PhD; Smita Kohli, MD; Mengjun Wang, MD; Souheila Hachem, BS; Kefei Zhang, MD

Background—Elamipretide (MTP-131), a novel mitochondria-targeting peptide, was shown to reduce infarct size in animals with myocardial infarction and improve renal function in pigs with acute and chronic kidney injury. This study examined the effects of chronic therapy with elamipretide on left ventricular (LV) and mitochondrial function in dogs with heart failure (HF).

Methods and Results—Fourteen dogs with microembolization-induced HF were randomized to 3 months monotherapy with subcutaneous injections of elamipretide (0.5 mg/kg once daily, HF+ELA, n=7) or saline (control, HF-CON, n=7). LV ejection fraction, plasma n-terminal pro-brain natriuretic peptide, tumor necrosis factor-α, and C-reactive protein were measured before (pretreatment) and 3 months after initiating therapy (post-treatment). Mitochondrial respiration, membrane potential (Δψm), maximum rate of ATP synthesis, and ATP/ADP ratio were measured in isolated LV cardiomyocytes obtained at post-treatment. In HF-CON dogs, ejection fraction decreased at post-treatment compared with pretreatment (29±1% versus 31±2%), whereas in HF+ELA dogs, ejection fraction significantly increased at post-treatment compared with pretreatment (36±2% versus 30±2%; P<0.05). In HF-CON, n-terminal pro-brain natriuretic peptide increased by 88±120 pg/mL during follow-up but decreased significantly by 774±85 pg/mL in HF+ELA dogs (P<0.001). Treatment with elamipretide also normalized plasma tumor necrosis factor-α and C-reactive protein and restored mitochondrial state-3 respiration, Δψm, rate of ATP synthesis, and ATP/ADP ratio (ATP/ADP: 0.38±0.04 HF-CON versus 1.16±0.15 HF+ELA; P<0.001).

Conclusions—Long-term therapy with elamipretide improves LV systolic function, normalizes plasma biomarkers, and reverses mitochondrial abnormalities in LV myocardium of dogs with advanced HF. The results support the development of elamipretide for the treatment of HF. (Circ Heart Fail. 2016;9:e002206. DOI: 10.1161/CIRCHEARTFAILURE.115.002206.)

Key Words: cardiopilin ■ heart failure ■ mitochondria ■ myocardial energetics ■ ventricular function
Methods
The canine model of intracoronary microembolization-induced chronic HF used in this study was previously described in detail.20 Fourteen healthy mongrel dogs, weighing between 20.8 and 25.7 kg, underwent serial intracoronary microembolizations performed 1 to 2 weeks apart to produce HF. Embolizations were discontinued when LV ejection fraction (EF), determined angiographically, was ≤30%. The study was approved by Henry Ford Health System Institutional Animal Care and Use Committee and conformed to the National Institute of Health Guide and Care for Use of Laboratory Animals (National Institutes of Health publication No. 85-23). Six weeks after the last microembolizations, HF dogs were randomized to 3 months therapy with subcutaneous injections of elamipretide (0.5 mg/kg once daily, n=7, HF+ELA) or vehicle (normal saline, once daily, n=7). Vehicle-treated HF dogs served as controls (HF-CON). Intravenous elamipretide has a half-life of 4 hours in dogs, and it is 100% renally excreted with 99% recovery in urine 24 hours after administration. Elamipretide’s peak plasma concentration is 325 ng/mL at 1 hour post dosing at 0.05 mg/kg. In all dogs, hemodynamic, ventriculographic, echocardiographic, and Doppler measurements were made at baseline, before treatment (pretreatment) and repeated 3 months after initiating treatment (post-treatment). For more details on the methods in this study, please see Data Supplement.

To gain further insight into the mechanism of action of elamipretide, we also examined the acute effects of intravenous elamipretide on LV function. In this acute study, 12 HF dogs were randomized to a 2 hour infusion of elamipretide (0.05 mg/kg per hour, n=7) or to a 2 hour infusion of v/v of normal saline (control, n=5).

Histomorphometric Measurements in LV Tissue
After the final hemodynamic study and while under general anesthesia, the dog’s chest was opened and the heart rapidly removed and LV tissue prepared for histological and biochemical evaluation. The volume fraction of replacement fibrosis and interstitial fibrosis, myocyte cross-sectional area, and section double-stained with rhodamine-labeled Griffonia Simplicifolia diomyocyte border to measure myocyte cross-sectional area, and section double-stained with rhodamine-labeled Griffonia Simplicifolia lectin I were used to measure capillary density and oxygen diffusion distance. Histomorphometrically as previously described.23,24 LV tissue from 7 normal dogs was used for comparisons. Tissue sections were stained with Gomori trichrome to identify fibrous tissue. Cryostat sections stained with fluorescein-labeled peanut agglutinin were used to delineate cardiomyocyte border to measure myocardial cross-sectional area, and sections double-stained with rhodamine-labeled Griffonia Simplicifolia lectin I were used to measure capillary density and oxygen diffusion distance, the latter defined as half the distance between 2 adjoining capillaries.

Determination of Mitochondrial Function
The following mitochondrial function measures were assessed in freshly collagenase-isolated, digitonin-permeabilized cardiomyocytes: mitochondrial state-3 respiration was measured using a Clark-type electrode (Strathclyde Respirometer).6 Mitochondrial membrane potential (Δψm) was measured using the commercially available JC-1 cationic fluorescent dye kit (Sigma, St. Louis, MO). This assay is an indicator of mitochondrial potential which, in presence and absence of valinomycin, exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green (495ex/534em) to red (495ex/590em).7 mPTP opening was assessed using calcine. The rate of calcine exit through mPTP was measured by recording the fluorescence signal every 2 minutes and calculated as a percent change from maximal fluorescence signal. Mitochondrial maximal rate of ATP synthesis and the ratio ATP/ADP were determined using the ApoSENSOR ADP/ATP ratio bioluminescent assay kit (BioVision, Milpitas, CA). This assay uses the enzyme luciferase to catalyze the formation of light from ATP and luciferin. The effects of an in vitro 1 hour incubation of isolated cardiomyocytes from 3 untreated HF dogs with varying concentrations of elamipretide (0.0, 0.01, 0.1, 1.0 μM) on mitochondrial state-3 respiration was also examined. ADP-stimulated respiration was determined in aliquots of 10 μL gravity-settled cardiomyocytes.

Determination of Mitochondrial Complex I and IV Activities
The activity of mitochondrial complex-I was assayed spectrophotometrically in mitochondrial membrane fractions obtained from LV anterior wall.25 Complex-I activity was calculated as the rotenone-sensitive NADH:ubiquinone oxidoreductase activity and expressed as nmoles/min per mg protein. The activity of mitochondrial complex-IV (cytochrome c oxidase) was determined polarographically.

2 Hours Intravenous Infusion Treatment Effect, Δ

<table>
<thead>
<tr>
<th></th>
<th>EDV (ml)</th>
<th>ESV (ml)</th>
<th>EF (%)</th>
<th>SV (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control Infusion</td>
<td>p=0.145</td>
<td>p=0.006</td>
<td>p=0.028</td>
<td></td>
</tr>
<tr>
<td>Elamipretide Infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3 Months Subcutaneous Treatment Treatment Effect, Δ

<table>
<thead>
<tr>
<th></th>
<th>EDV (ml)</th>
<th>ESV (ml)</th>
<th>EF (%)</th>
<th>FAS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF-CON</td>
<td>p=0.08</td>
<td>p=0.001</td>
<td>p=0.05</td>
<td></td>
</tr>
<tr>
<td>HF+ELA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Top, Change Δ (treatment effect) between pretreatment and 2 hour intravenous infusion of elamipretide on left ventricular (LV) end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), and stroke volume (SV) in untreated heart failure control dogs (HF-CON) and heart failure dogs treated with elamipretide (HF+ELA). Bottom, Change Δ (treatment effect) between pretreatment and 12 weeks post-treatment for LV EDV, ESV, EF, and fractional area of shortening (FAS) in HF-CON and HF+ELA dogs. Probability values are comparisons between HF-CON and HF+ELA dogs. Statistical significance based on t-statistic for 2 means. All bar graphs are depicted as mean±SEM.
in mitochondrial membrane fractions and expressed as nmols molecular oxygen/min per mg protein.

### Determination of Abundances of Key Subunits of Complex-I, -II, -III, -IV, and -V

Abundance of key subunits of mitochondrial complexes was determined by Western blotting using the Total OXYPHOS Antibody Cocktail ab110413 (abcam, Cambridge, MA) and bands quantified in densitometric units (du). The subunits were as follows: complex-I subunit NDUFB8; complex-II succinate dehydrogenase subunit B; complex-III subunit Core 2; complex-IV subunit I, and complex-V ATP synthase subunit a.

### Western Blotting and Measurements of Cardiolipin and ROS

Western blotting was used to quantify changes in LV tissue levels of specific mitochondrial functions/dynamics and signaling proteins. Western blots were performed using primary antibodies and horse radish peroxidase–coupled secondary antibodies. Protein bands were visualized by chemiluminescence reagents (Thermo Scientific, Pittsburg, PA). Proteins included endothelial nitric oxide synthase, sarcoplasmic reticulum Ca2+-ATPase (SERCA-2a), and receptor coactivator-1α, cytosolic cytochrome c, active caspase 3, P53, and ROS.

### Table 1. Hemodynamic, Ventriculographic, Echocardiographic, and Plasma Biomarker Measures in Untreated Heart Failure Control Dogs (HF-CON; n=7) and Heart Failure Dogs Treated With Elamipretide (HF+ELA; n=7)

<table>
<thead>
<tr>
<th>Measure</th>
<th>HF-CON Baseline</th>
<th>HF-CON Pretreatment</th>
<th>HF-CON Post-Treatment</th>
<th>HF+ELA Baseline</th>
<th>HF+ELA Pretreatment</th>
<th>HF+ELA Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>22.7±0.7</td>
<td>23.1±0.7</td>
<td>24.0±0.5</td>
<td>21.5±0.4</td>
<td>22.1±0.4</td>
<td>23.0±0.7</td>
</tr>
<tr>
<td>HR, beats per min</td>
<td>86±2</td>
<td>88±2</td>
<td>84±1</td>
<td>90±3</td>
<td>82±2*</td>
<td>84±3</td>
</tr>
<tr>
<td>mAoP, mm Hg</td>
<td>72±1</td>
<td>74±1</td>
<td>74±1</td>
<td>75±1</td>
<td>73±2</td>
<td>71±1</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>12±1</td>
<td>15±1*</td>
<td>16±1*</td>
<td>12±1</td>
<td>17±1*</td>
<td>14±0.3†</td>
</tr>
<tr>
<td>LVEDV, mL</td>
<td>57±3</td>
<td>63±3</td>
<td>69±5*</td>
<td>62±2</td>
<td>75±4*</td>
<td>77±3*</td>
</tr>
<tr>
<td>LVESV, mL</td>
<td>28±1</td>
<td>44±3*</td>
<td>48±4*</td>
<td>32±1</td>
<td>52±2*</td>
<td>49±2*</td>
</tr>
<tr>
<td>LV EF, %</td>
<td>51±1</td>
<td>31±1*</td>
<td>29±1*</td>
<td>49±1</td>
<td>30±2*</td>
<td>36±2†</td>
</tr>
<tr>
<td>SV, mL</td>
<td>29±2</td>
<td>19±1*</td>
<td>21±2*</td>
<td>31±1</td>
<td>23±2*</td>
<td>28±2</td>
</tr>
<tr>
<td>CO, L/min</td>
<td>2.5±0.16</td>
<td>1.7±0.11*</td>
<td>1.76±0.15*</td>
<td>2.73±0.08</td>
<td>1.86±0.14*</td>
<td>2.34±0.11†</td>
</tr>
<tr>
<td>CI, L/min per m²</td>
<td>3.2±0.2</td>
<td>2.1±0.1*</td>
<td>2.1±0.2*</td>
<td>3.3±0.1</td>
<td>2.2±0.2*</td>
<td>2.8±0.1*</td>
</tr>
<tr>
<td>SVR, dynes s/cm²</td>
<td>2809±156</td>
<td>4223±232*</td>
<td>3855±274*</td>
<td>2642±105</td>
<td>3647±326*</td>
<td>2916±167†</td>
</tr>
<tr>
<td>EUI/Ai</td>
<td>7.5±0.9</td>
<td>4.5±0.4*</td>
<td>4.2±0.3*</td>
<td>7.3±1.0</td>
<td>4.8±0.6*</td>
<td>5.3±0.6*</td>
</tr>
<tr>
<td>DT, ms</td>
<td>102±2</td>
<td>79±4*</td>
<td>73±5*</td>
<td>102±4</td>
<td>75±5*</td>
<td>84±4*</td>
</tr>
<tr>
<td>LV EDWS, gm/cm²</td>
<td>46±2</td>
<td>77±3*</td>
<td>78±9*</td>
<td>47±3</td>
<td>87±9*</td>
<td>69±7*</td>
</tr>
<tr>
<td>rt-Pro BNP, pg/mL</td>
<td>262±15</td>
<td>1163±126*</td>
<td>1250±113*</td>
<td>278±17</td>
<td>1128±72*</td>
<td>354±29†</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>1.29±0.14</td>
<td>3.85±0.16*</td>
<td>3.95±0.23*</td>
<td>1.24±0.13</td>
<td>4.36±0.09*</td>
<td>1.44±0.09†</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>9.1±2.3</td>
<td>24.9±2.1*</td>
<td>28.6±1.9†</td>
<td>10.1±1.1</td>
<td>26.2±2.2*</td>
<td>12.0±0.3†</td>
</tr>
<tr>
<td>CRP, μg/mL</td>
<td>1.30±0.16</td>
<td>7.38±0.65*</td>
<td>8.99±1.36*</td>
<td>1.69±0.19</td>
<td>6.89±0.70*</td>
<td>1.72±0.29†</td>
</tr>
<tr>
<td>Plasma ROS (RLU×10⁵/mL)</td>
<td>5.0±0.3</td>
<td>21.1±3.1*</td>
<td>23.8±2.0*</td>
<td>5.3±0.5</td>
<td>20.7±2.6*</td>
<td>10.5±0.8*†</td>
</tr>
</tbody>
</table>

Table 2. Treatment Effect (Δ) in Untreated Heart Failure Control Dogs (HF-CON; n=7) and in Elamipretide-Treated Heart Failure Dogs (HF+ELA; n=7)

<table>
<thead>
<tr>
<th>Measure</th>
<th>HF-CON Value</th>
<th>HF+ELA Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔBody weight, kg</td>
<td>0.8±0.5</td>
<td>0.9±0.3</td>
<td>0.87</td>
</tr>
<tr>
<td>ΔHR, beats per min</td>
<td>−3.7±2.1</td>
<td>1.7±2.3</td>
<td>0.11</td>
</tr>
<tr>
<td>ΔmAoP, mm Hg</td>
<td>0.1±1.6</td>
<td>−1.3±1.6</td>
<td>0.54</td>
</tr>
<tr>
<td>ΔLVEDP, mm Hg</td>
<td>0.6±0.6</td>
<td>−2.3±0.5</td>
<td>0.003</td>
</tr>
<tr>
<td>ΔSV, mL</td>
<td>2.1±1.5</td>
<td>5.3±1.2</td>
<td>0.13</td>
</tr>
<tr>
<td>ΔCO, L/min</td>
<td>0.10±0.14</td>
<td>0.48±0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>ΔCI, L/m²</td>
<td>0.1±0.2</td>
<td>0.6±0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>ΔSVR, dynes s/cm²</td>
<td>−367±285</td>
<td>−781±228</td>
<td>0.28</td>
</tr>
<tr>
<td>ΔEUI/Ai</td>
<td>−0.3±0.2</td>
<td>0.4±0.7</td>
<td>0.36</td>
</tr>
<tr>
<td>ΔDT, ms</td>
<td>−5.7±6.5</td>
<td>8.4±5.1</td>
<td>0.11</td>
</tr>
<tr>
<td>ΔEDEWS, g/cm²</td>
<td>0.9±9.0</td>
<td>−17.6±3.4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

At indicates time–velocity integral representing left atrial contraction; Cl, cardiac index; CO, cardiac output; CRP, C-reactive protein; DT, deceleration time of early mitral inflow velocity; EUI, end-diastolic pressure; EDV, end-diastolic volume; EDWS, LV end-diastolic circumferential wall stress; EF, ejection fraction; Ei, time–velocity integral of the mitral inflow velocity waveform representing early filling; Ei/Ai, ratio of Ei to Ai; ESE, end-systolic volume; HR, heart rate; IL, interleukin; LV, left ventricular; mAoP, mean aortic pressure; n/Pro-BNP, n-terminal-pro brain natriuretic peptide; RLU, relative light units; ROS, level of reactive oxygen species in plasma; SV, stroke volume; SVR, systemic vascular resistance; and TFF-α, tumor necrosis factor-α. All data are mean±SEM. *P<0.05 vs baseline. †P<0.05 vs pretreatment.
and capable of binding to proteins and forming stable adducts, was also measured using the commercially available Oxiselect HNE-His Adduct ELISA Kit (Biolabs, Inc, San Diego, CA).

**Statistical Analysis**

Within-group comparisons of hemodynamic, ventriculographic, echocardiographic, Doppler, and plasma biomarker measures were made using repeated measures analysis of variance with alpha set at 0.05. If significance was attained, pairwise comparisons between baseline, pretreatment, and post-treatment measures were made using the Student–Neuman–Kuels test with $P<0.05$ considered significant. To assess treatment effect, the change ($\Delta$) in each measure from pre-treatment to post-treatment within each study arm was calculated and the $\Delta$s compared between the 2 groups using a t-statistic for 2 means with $P<0.05$ considered significant. Histological and biochemical measures between normal, HF-CON, and HF+BEN dogs were compared using 1-way analysis of variance with alpha set at 0.05. If significance was attained by analysis of variance, pairwise comparisons were performed using the Student–Neuman–Kuels test with $P<0.05$ considered significant. All the data exhibited normal distributions, and nonparametric testing led to similar results. Data are reported as means±standard error of the mean.

**Results**

**Effects of Acute Intravenous Infusion of Elamipretide**

Compared with intravenous saline, intravenous elamipretide had no effect on heart rate (HR), mean aortic pressure (mAoP), or systemic vascular resistance (SVR). Elamipretide had no effect on LV end-diastolic volume but significantly decreased end-systolic volume and significantly increased EF and stroke volume (Figure 1).

**Chronic Studies With Subcutaneous Elamipretide**

**Within-Group Changes in Hemodynamics and Plasma Biomarkers**

Hemodynamic, ventriculographic, echocardiographic, Doppler, and plasma biomarker results are shown in Table 1. There were no significant differences between the 2 study groups in any measures obtained at baseline or at pretreatment. In the HF-CON, HR, mAoP, LV end-diastolic pressure, stroke volume, cardiac output, cardiac index, SVR, ratio of time–velocity integral of early mitral inflow velocity and time–velocity integral during left atrial contraction (Ei/Ai), deceleration time of early mitral inflow velocity, and LV circumferential end-diastolic wall stress did not change significantly over the course of 3 months of follow-up compared with pretreatment values (Table 1). In the HF+ELA, HR and mAoP were also not significantly changed during therapy. In this group, Ei/Ai and deceleration time of early mitral inflow velocity tended to increase but not significantly compared with pretreatment values (Table 1). Therapy with elamipretide significantly increased stroke volume, cardiac output, and cardiac index and decreased end-diastolic pressure, SVR, and end-diastolic wall stress.

In HF-CON, end-diastolic volume and end-systolic volume tended to increase and EF tended to decrease at post-treatment compared with pretreatment (Table 1). In HF+ELA, end-diastolic volume was unchanged, end-systolic volume tended to decrease, and EF increased significantly (Table 1). Plasma levels of n-terminal pro-brain natriuretic peptide, interleukin-6, tumor necrosis factor-$\alpha$, C-reactive protein, and ROS significantly increased in HF-CON compared with normal dogs. Treatment with elamipretide significantly reduced levels of all 5 biomarkers to near normal levels. In HF-CON, plasma levels of all 5 biomarkers remained markedly elevated throughout the treatment phase (Tables 1).

**Between-Group Changes in Hemodynamics and Plasma Biomarkers (Treatment Effect)**

Between-group comparisons of the change ($\Delta$) between pre-treatment and post-treatment measurements are shown in Table 2 and Figure 1. Compared with HF-CON, long-term therapy with elamipretide had no effect on HR, mAoP, and SVR but tended to increase stroke volume and decrease end-diastolic volume and end-systolic volume. Treatment with

| Table 3. Histomorphometric Findings at the End of 3 Months in Normal Dogs (n=7), in Untreated Heart Failure Control Dogs (HF-CON; n=7), and in Heart Failure Dogs Treated With Elamipretide (HF+ELA; n=7) |

<table>
<thead>
<tr>
<th>VFRF, %</th>
<th>VFRF, %</th>
<th>CD, cap/mm²</th>
<th>ODD, μm</th>
<th>MCSA, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.7±0.07</td>
<td>2609±79.5</td>
<td>8.9±0.17</td>
<td>410±10.0</td>
</tr>
<tr>
<td>HF-CON</td>
<td>12.3±0.6*</td>
<td>11.8±0.5*</td>
<td>1806±80*</td>
<td>12.6±0.2*</td>
</tr>
<tr>
<td>HF+ELA</td>
<td>11.2±0.9</td>
<td>9.7±0.5†</td>
<td>2026±127†</td>
<td>11.4±0.4†</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. CD indicates capillary density; MCSA, myocyte cross-sectional area; ODD, oxygen diffusion distance; VFRF, volume fraction of interstitial fibrosis; and VRFIF, volume fraction of replacement fibrosis. All data are mean±SEM.

*P<0.05 vs normal.
†P<0.05 vs HF-CON.
Elamipretide significantly increased EF, cardiac output, cardiac index, and LV fractional area of shortening. Measures of LV diastolic function also tended to improve with elamipretide as evidenced by a decrease of end-diastolic pressure and end-diastolic wall stress and a trend for an increase in Ei/Ai and deceleration time of early mitral inflow velocity. Compared with HF-CON, elamipretide significantly decreased plasma levels of n-terminal pro-brain natriuretic peptide, interleukin-6, tumor necrosis factor-α, C-reactive protein, and ROS to near normal levels (Figure 2).

Histomorphometric Findings and Tissue ROS
Compared with normal dogs, HF-CON dogs showed significant increases in volume fraction of replacement fibrosis, volume fraction of interstitial fibrosis, oxygen diffusion distance, and myocyte cross-sectional area and a decrease in capillary density (Table 3). Elamipretide significantly reduced volume fraction of interstitial fibrosis, oxygen diffusion distance, and myocyte cross-sectional area and increased capillary density. The volume fraction of replacement fibrosis also tended to decrease but did not reach statistical significance. Total ROS in LV tissue was significantly higher in HF-CON compared with normal dogs and decreased to near normal in HF+ELA (Table 4). Levels of 4-hydroxynonenal adducts were

<table>
<thead>
<tr>
<th>Elamipretide</th>
<th>Normal</th>
<th>HF-CON</th>
<th>HF+ELA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV tissue ROS (RLU x 10^4/μg protein)</td>
<td>0.77±0.04</td>
<td>1.45±0.18*</td>
<td>0.90±0.06*†</td>
</tr>
<tr>
<td>4-HNE adducts, ng/mg protein</td>
<td>185±21</td>
<td>399±35*</td>
<td>252±18*†</td>
</tr>
<tr>
<td>Complex-I subunit, du</td>
<td>0.51±0.02</td>
<td>0.15±0.01*</td>
<td>0.29±0.03*†</td>
</tr>
<tr>
<td>Complex-II subunit, du</td>
<td>0.45±0.06</td>
<td>0.14±0.01*</td>
<td>0.34±0.02*†</td>
</tr>
<tr>
<td>Complex-III subunit, du</td>
<td>0.40±0.07</td>
<td>0.16±0.02*</td>
<td>0.27±0.03*†</td>
</tr>
<tr>
<td>Complex-IV subunit, du</td>
<td>0.61±0.04</td>
<td>0.26±0.05*</td>
<td>0.44±0.03*†</td>
</tr>
<tr>
<td>Complex-V subunit, du</td>
<td>4.97±0.32</td>
<td>2.12±0.37*</td>
<td>2.81±0.50*†</td>
</tr>
</tbody>
</table>

4-HNE indicates 4-hydroxynonenal; MITO, mitochondrial; RLU, relative luminescence units; and ROS, reactive oxygen species. All data are means±SEM.

*P<0.05 vs normal.
†P<0.05 vs HF-CON.

Table 4. ROS and 4-HNE Levels and MITO Complexes I, II, III, IV, and V Abundances in LV Myocardium of Normal Dogs (n=6), Untreated Heart Failure Control Dogs (HF-CON; n=7), and Elamipretide-Treated Heart Failure Dogs (HF+ELA; n=7)

Figure 3. Mitochondrial function in cardiomyocytes of left ventricular myocardium of normal dogs, untreated heart failure control dogs (HF-CON), and dogs with heart failure treated with elamipretide (HF+ELA). Top Left, Mitochondrial state 3 and 4 respiration. Top Right, Mitochondrial membrane potential. Bottom Left, Mitochondrial permeability transition pore (mPTP). Bottom Right, Maximum rate of adenosine triphosphate (ATP) synthesis and ratio of ATP to adenosine diphosphate (ADP). Probability values are comparisons between normal dogs, HF-CON, and HF-ELA dogs. Statistical significance based on 1-way analysis of variance (ANOVA) followed by the Student–Neuman–Keuls. All bar graphs are depicted as mean±SEM.
Mitochondrial Function
Mitochondrial state-3 respiration, Δψm, maximum rate of ATP synthesis, and ATP/ADP ratio were all decreased in HF-CON compared with normal dogs (Figure 3). Treatment with elamipretide significantly increased all measures to near normal levels (Figure 3). The rate of calcein exit was significantly slower in HF+ELA dogs compared with HF-CON (Figure 3), indicative of reduced opening of the mPTP. Complex-I and complex-IV activities were significantly reduced in HF-CON compared with normal dogs (Figure 4). Elamipretide significantly increased enzymatic activities of both complexes to near normal levels (Figure 4). In all instances, the observed improvements in mitochondrial function parameters after treatment with elamipretide trended toward baseline (normal state) and at no time exceeded normal levels, indicating that full recovery of mitochondrial function was not necessary to achieve the degree of observed hemodynamic improvement.

In vitro incubation of isolated cardiomyocytes from untreated HF dogs with increasing concentration of elamipretide showed a concentration-dependent improvement in state-3 respiration. In the absence of elamipretide, ADP-stimulated respiration was 248±9 nAtom O/min per mg protein and increased to 303±33 at 0.1 μmol/L, to 371±28 at 1.0 μmol/L, and to 405±39 at 0.1 μmol/L, to 371±28 at 1.0 μmol/L concentration of elamipretide.

Proteins That Modulate Mitochondrial Function
There were no differences in protein level of the internal control β-actin between normal dogs (0.44±0.05 du) and HF-CON (0.41±0.04 du) or elamipretide-treated HF dogs (0.45±0.04 du). Proteins that regulate mitochondrial biogenesis and respiration were dysregulated in HF-CON compared with normal dogs (Figure 5). Compared with normal, levels of endothelial nitric oxide synthase and peroxisome proliferator–activated receptor coactivator-1α were significantly decreased in HF-CON, whereas inducible nitric oxide synthase was significantly upregulated. Elamipretide normalized levels of both nitric oxide synthase isofoms and peroxisome proliferator–activated receptor coactivator-1α were significantly increased in HF-CON, whereas inducible nitric oxide synthase was significantly upregulated. Elamipretide normalized levels of both nitric oxide synthase isofoms and peroxisome proliferator–activated receptor coactivator-1α were significantly decreased in HF-CON, whereas inducible nitric oxide synthase was significantly upregulated.

Cytosolic levels of cytochrome c were significantly increased in HF-CON as were tissue levels of active caspase-3. Treatment with elamipretide normalized the expression of both proapoptotic proteins (Figure 5). Compared with normal, HF-CON dogs had significantly lower levels of SERCA-2a, which was significantly increased to near normal by elamipretide (Figure 5). Total cardiolipin and total cardiolipin normalized to mitochondrial protein were significantly decreased in LV of HF-CON compared with normal dogs as were the levels of total (18:2)CL subspecies and total (18:2)CL subspecies normalized to mitochondrial protein. Treatment with elamipretide significantly increased all measures to near normal levels (Figure 6). Protein levels of key subunits of mitochondrial complexes I–V decreased significantly in HF dogs compared with normal dogs (Figure 7 and Table 4). Treatment with elamipretide partially restored normal levels of all key subunits. Compared with untreated controls, levels of subunits of complexes I, II, and IV improved significantly after long-term therapy with elamipretide, whereas the observed improvement in subunits of complexes III and V did not reach statistical significance (Figure 7 and Table 4). In all instances, the observed improvements in protein levels after treatment with elamipretide trended toward baseline (normal state) and at no time exceeded normal levels.

Discussion
The results indicate that long-term monotherapy with elamipretide improves LV function and prevents progressive LV enlargement. The magnitude of improvement is similar to that observed in this canine HF model after long-term therapy with angiotensin-converting enzyme inhibitors and β-blockers.27 Improved LV function was associated with reductions in plasma biomarkers of natriuretic peptides and proinflammatory cytokines and was not associated with significant changes in HR, mAoP, or SVR. These benefits occurred alongside normalization of mitochondrial function evidenced by improved rate of ATP synthesis and reduced ROS formation. Elamipretide also attenuated structural LV remodeling evidenced by reduction of cardiomyocyte hypertrophy and interstitial fibrosis and increased capillary

![Complex-I Activity](image)

**Complex-I Activity (nmol/min/mg protein)**

![Complex-IV Activity](image)

**Complex-IV Activity (nmol oxygen /min/mg protein)**

Figure 4. Bar graphs (means±SEM) depicting Complex-I (Top) and Complex-IV (Bottom) enzymatic activity in left ventricular (LV) myocardium of normal (NL) dogs, untreated heart failure control dogs (HF-CON), and dogs with heart failure treated with elamipretide (HF+ELA). Statistical significance based on 1-way analysis of variance (ANOVA) followed by the Student–Neuman–Keuls.
Normalization of these structural components likely promotes greater LV compliance and tissue oxygenation and hence improved passive LV filling and overall LV function. The improvements in histomorphometric measure of structural remodeling after treatment with elamipretide are similar in magnitude to those seen in this canine HF model after monotherapy with AT1 receptor antagonists and β-blockers. In this study, chronic treatment with elamipretide improved energy availability in the failing myocardium evidenced by increased maximum rate of ATP synthesis and increased ATP/ADP ratio. In addition to improving mitochondrial state-3 respiration and mitochondrial membrane potential and reducing opening of the mPTP, treatment with elamipretide also normalized the activity of complex-I and complex-IV while limiting the formation of ROS. Improved ATP synthesis by mitochondria can also lead to improved sarcoplasmic reticulum calcium cycling by increasing SERCA-2a activity. Reduced SERCA-2a activity and expression are key maladaptations in HF. Treatment with elamipretide was associated with improved protein levels of SERCA-2a.

The improvement in mitochondrial function after long-term therapy with elamipretide leads to normalization of cardiolipin. The latter is likely facilitated by the ability of elamipretide to permeate the outer mitochondrial membrane and bind to cardiolipin, a signature phospholipid of the inner mitochondrial membrane that plays an important role in cristae formation, activity of respiratory complexes, organization of the respiratory complexes into supercomplexes for oxidative phosphorylation, mitochondrial fusion, and mitochondrial DNA stability and segregation. Cardiolipin is biosynthesized in a series of steps from phosphatidic acid and remodeled into a form which contains 4 18:2 fatty acid chains, (18:2)4CL. Cardiolipin peroxidation and depletion have been reported in a variety of pathological conditions associated with energy deficiency. In the present study, total cardiolipin and (18:2)4CL were decreased in LV myocardium of dogs with HF and treatment with elamipretide normalized total cardiolipin and (18:2)4CL. The decrease in cardiolipin was driven by changes in the lipid structure on the inner mitochondrial membrane because of peroxidation and not necessarily a reflection of changes in the total LV myocardial pool of cardiolipin.

Figure 5. Bar graphs (mean±SEM) depicting protein levels of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), cytosolic cytochrome c (CCC), active caspase 3, and sarcoplasmic reticulum (SR) Ca2+-ATPase (SERCA-2a) in left ventricular (LV) myocardium of normal dogs (NL), untreated failure control dogs (HF-CON), and heart failure dogs treated with elamipretide (HF+ELA). Below each graph are bands from Western Blots of each protein. du indicates densitometric units. Statistical significance based on 1-way analysis of variance (ANOVA) followed by the Student–Neuman–Keuls.
mitochondrial protein. This observation is also supported by results showing concordant changes in LV myocardial levels of 4-hydroxynonenal, a major product of lipid peroxidation. Nevertheless, additive beneficial effects of elamipretide on mitochondrial biogenesis/turnover may also coexist alongside membrane remodeling. Cardiolipin is essential for activity of mitochondrial complexes and, in particular, complex I and complex-IV. Defects of complex I are integral to the formation of ROS, whereas complex-IV is essential for oxidative phosphorylation. In the present study, elamipretide normalized the activity of complex-I and -IV, a finding consistent with normalization of cardiolipin. Elamipretide also restored, albeit in part, levels of key subunits of mitochondrial complexes I through V and reduced the formation of ROS in LV myocardium. Preserving cardiolipin is also important in respiratory function through supercomplex assembly. Even though supercomplex expression was not measured in the present study, others have shown that elamipretide preserves supercomplex-dependent mitochondrial function in LV myocardium of rats subjected to ischemia–reperfusion injury.32

Even though the primary objective of this study was to evaluate the chronic effects of elamipretide, we also showed that a short-term (2 hours) intravenous infusion of elamipretide improved LV systolic function and that in vitro incubation of failing canine cardiomyocytes in increasing concentration of elamipretide elicits improvement in ADP-dependent (state-3) mitochondrial respiration. These results indicate that elamipretide can elicit short-term benefits on LV function possibly through improved mitochondrial function as evidence by improved respiration. The data also suggest that an improvement in the abundance of 18:2 cardiolipin is not required in the short term for enhancement of mitochondrial function by elamipretide. It is not likely that novel synthesis and remodeling of cardiolipin can occur in a short 2 hour span of elamipretide infusion. It is possible, although remains to be shown, that the electrostatic interaction between elamipretide

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**Figure 6.** Bar graphs (mean±SEM) depicting total cardiolipin (CL) level (Top Left) and total cardiolipin level normalized to mitochondrial (MITO) protein (Top Right) and total (18:2)_4 cardiolipin level (Bottom Left) and total (18:2)_4 cardiolipin level normalized to mitochondrial protein level (Bottom Right) in left ventricular (LV) myocardium of normal (NL) dogs, untreated heart failure control dogs (HF-CON), and dogs with heart failure treated with elamipretide (HF+ELA). Statistical significance based on 1-way analysis of variance (ANOVA) followed by the Student–Neuman–Keuls.

**Figure 7.** Western blots of protein abundances of key mitochondrial subunits of mitochondrial complexes I, II, III, IV, and V performed using Total OXPHOS Western Blot Antibody Cocktail ab110413. The blot depicts results from 2 normal dogs (NL), 2 untreated heart failure control dogs (HF-CON), and 2 dogs with heart failure treated with elamipretide (HF+ELA). The specific subunits are listed in the Methods section.
and cardiolipin is itself sufficient to stabilize the complexes on the electron transport chain and, in doing so, improve efficiency of the electron transfer culminating in reduced ROS and enhanced oxidative phosphorylation. This explanation is supported by studies of old mice with mitochondrial dysfunction, in which resting and maximal mitochondrial ATP production and cell energy state (phosphocreatine/ATP) were rapidly reversed only 1 hour after injection of elamipretide. Mitochondria are a major source of ROS production. Excess ROS formation can lead to tissue injury, cardiomyocyte degeneration, and programmed cell death. Increased ROS can contribute to increased opening of the mPTP and has been shown to interact with calcium signaling and several ion channels involved in cardiac hypertrophy signaling and its progression to HF. We previously showed that HF is associated with opening of the mPTP with an attendant increase in the level of cytochrome c in the cytosol and consequently an increase in cardiomyocyte apoptosis. In the present study, long-term therapy with elamipretide reduced ROS formation, attenuated mPTP openings, and significantly decreased the levels of cytosolic cytochrome c and active caspase-3, thus suppressing a major signaling pathway for apoptosis. Levels of plasma proinflammatory cytokines were reduced in the present study after chronic treatment with elamipretide. We are not aware of any evidence that elamipretide acts directly as an anti-inflammatory. It is more likely that by reducing excessive ROS formation, elamipretide limits tissue injury and, in doing so, attenuates proinflammatory cytokines.

In conclusion, this is the first study to our knowledge where the effects of long-term therapy with elamipretide were evaluated in a chronic model of HF. The results support the hypothesis that targeting mitochondrial dysfunction in HF is key to improving overall LV function. Elamipretide represents a new class of compounds that can improve the availability of energy to failing heart and reduce the burden of tissue injury caused by excessive ROS production. In doing so, elamipretide limits end-organ damage and ensures the production of energy commensurate with the needs of the myocardium for improving cell function and overall LV performance.

**Sources of Funding**

This study was supported, in part, by a research grant from Stealth Biotherapeutics, Inc and by the National Heart, Lung, and Blood Institute PO1 HL074237-10.

**Disclosures**

Dr. Sabbah has received research grants from Stealth Biotherapeutics, Inc and is a consultant for Stealth Biotherapeutics. The other authors report no conflicts.

**References**

Structural and functional abnormalities exist in constituent mitochondria of the failing heart and can lead to excessive production of reactive oxygen species and, importantly, to reduced rate of ATP synthesis, the energy currency of living cells, and the lifeline of cardiomyocytes. Excessive formation of reactive oxygen species results in tissue injury, whereas reduced rate of ATP synthesis contributes to cellular dysfunction with both contributing to progressive worsening of left ventricular function that characterizes the heart failure (HF) state. The absence of drugs that can prevent, reverse, or even limit mitochondrial dysfunction in HF has contributed to the lack of research exploring the potential therapeutic merits of reversing mitochondrial dysfunction in HF. Current HF drugs elicit their benefits largely by reducing cardiac workload that leads to reduced myocardial energy demands to levels in-line with the existing limited energy supply. This preclinical study in dogs with chronic HF describes the effects of a novel, first-in-class mitochondria targeting peptide, elamipretide, that normalizes mitochondrial energetics by sustaining respiratory supercomplexes (Abst). Circ Res. 2014;115:A337.

CLINICAL PERSPECTIVE


Elamipretide in Heart Failure

Sabbah et al
Chronic Therapy With Elamipretide (MTP-131), a Novel Mitochondria-Targeting Peptide, Improves Left Ventricular and Mitochondrial Function in Dogs With Advanced Heart Failure

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Circ Heart Fail. 2016;9:e002206
doi: 10.1161/CIRCHEARTFAILURE.115.002206
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/9/2/e002206

Data Supplement (unedited) at:
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Chronic Therapy with Elamipretide (MTP-131), a Novel Mitochondria-Targeting Peptide, Improves Left Ventricular and Mitochondrial Function in Dogs with Advanced Heart Failure

SUPPLEMENTAL MATERIAL

Methods
In this study, 14 healthy mongrel dogs, underwent serial intracoronary microembolizations performed 1 to 2 weeks apart, to produce HF. Embolizations were discontinued when LV ejection fraction (EF), determined angiographically, was ~30%. Nearly 6 weeks after the last microembolizations, dogs were randomized to 3 months of therapy with subcutaneous injections with elamipretide (0.5 mg/kg per day, n=7) or saline (Control, n=7). All the procedures were performed during cardiac catheterization under general anesthesia and sterile conditions. Induction of anesthesia was initiated with intravenous hydromorphone (0.22 mg/kg) and diazepam (0.17 mg/kg). Plane of anesthesia was maintained with 1-2% isoflurane.

Hemodynamic, Ventriculographic and Echocardiographic/Doppler Measurements
Aortic and LV pressures were measured with catheter-tip micromanometers (Millar Instruments, Houston, TX). Left ventriculograms were obtained with the dog placed on its right side and recorded on digital media at 30 frame/sec during the injection of 20 ml of contrast material (ISOVU-300, Bracco Diagnostics, Inc., Princeton, NJ). Correction for image magnification was made with a radiopaque calibrated markers imbedded in the shaft of the LV ventriculography catheter. LV end-systolic volume (ESV), end-diastolic
volume (EDV) and LV EF were calculated using the area-length method (1). Stroke volume was calculated as the difference between EDV and ESV. Cardiac output (CO) was calculated as the product of stroke volume (SV) and heart rate (HR). Systemic vascular resistance (SVR) was calculated as previously described (2). Cardiac index (CI) was measured as the ratio of CO to body surface area.

Echocardiographic and Doppler studies were performed using a General Electric VIVID-7 ultrasound system with a 3.5 MHZ transducer and recorded on digital media for off-line analysis. Trans-mitral inflow velocity waveforms, measured using pulsed-wave Doppler echocardiography, were used to calculate indexes of LV diastolic function namely the time-velocity integral of mitral inflow velocity waveform representing early filling (Ei), the time-velocity integral representing LA contraction (Ai), the ratio Ei/Ai, and deceleration time (DT) of early mitral inflow velocity as previously described (2, 3). LV end-diastolic circumferential wall stress (EDWS) was calculated as previously described (2).

**Determination of Plasma Biomarkers and Reactive Oxygen Species**

Assay kits for TNFα and IL-6 were purchased from R&D Systems, Inc, Minneapolis, MN; nt-pro-BNP kits from Kamiya Biomedical Company, Seattle, WA, and CRP kits from ALPCO, Salem, NH. Detection method for all these kits were colorimetric and plasma volume used in TNF and IL6 assays were 150 µl, in nt-proBNP 35 µl, and CRP 100 µl after 1000 fold dilution. Incubation time with primary antibody was overnight at 4°C except 10 min at room temperature in case of CRP. All the values calculated for the
biomarkers were from the standard curve generated using a software MasterPlex ReaderFit 2010. All the values of biomarkers were expressed per ml plasma without any normalization.

Total burden of reactive oxygen species (ROS) in plasma was determined in an aliquot of 100 µl of 10-folds diluted EDTA-plasma with PBS buffer using the luminol-dependent chemiluminescence assay and expressed as relative luminescence units (RLU)/ml (4). Total ROS in LV tissue was determined using 100 µl of 30 fold-diluted cytosol of 0.05% LV tissue using homogenate in PBS buffer with the same assay as plasma and expressed as RLU/mg protein (4).

**Histomorphometric Measurements**

The volume fraction of replacement fibrosis (VFRF), interstitial fibrosis (VFIF), myocyte cross-sectional area (MCSA), a measure of cardiomyocyte hypertrophy, capillary density (CD), and oxygen diffusion distance (ODD) were assessed histomorphometrically as previously described (5, 6). Briefly, from each heart, 3 transverse slices (3 mm thick), 1 each from the basal, middle, and apical thirds of the LV, were obtained. From each slice, transmural tissue blocks were obtained and embedded in paraffin blocks. From each block, 6 µm-thick sections were prepared and stained with Gomori trichrome to identify fibrous tissue. The VFRF, namely, the proportion of scar tissue to viable tissue in all 3 transverse LV slices, was calculated as the percent total surface area occupied by fibrous tissue by use of computer-based video densitometry (MOCHA, Jandel Scientific). LV free-wall tissue blocks were obtained from a second midventricular transverse slice,
mounted on cork with Tissue-Tek embedding medium (Sakura), and rapidly frozen in isopentane precooled in liquid nitrogen and stored at -70°C until used. Cryostat sections were prepared and stained with fluorescein-labeled peanut agglutinin (Vector Laboratories Inc) after pretreatment with 3.3 U/mL neuraminidase type V (Sigma Chemical Co) to delineate the myocyte border and the interstitial space, including capillaries. Sections were double stained with rhodamine-labeled Griffonia Simplicifolia lectin I (GSL-I) to identify capillaries. Average MCSA area was calculated by computer-assisted planimetry. The VFIF was calculated as the percent total surface area occupied by interstitial space minus the percent total area occupied by capillaries. CD density was calculated as the number of capillaries per square millimeter and ODD was calculated as half the distance between 2 adjoining capillaries.

Determination of Mitochondrial Function

Isolation of Cardiomyocytes: At the end of 3 months of therapy, cardiomyocytes were freshly isolated from LV free wall using collagenase as previously described (7, 8). The yield of rod-shaped cardiomyocytes that exclude tryptan blue was in the range of 80% to 90%.

Mitochondrial Respiration: Oxygen consumption was measured in freshly isolated, digitonin-permeabilized, cardiomyocytes using a Clark-type electrode (Strathklein Respirometer) in a final volume of 0.5 mL of respiration buffer as previously described (9). Mitochondrial (MITO) state-4 respiration was measured by adding substrate
(pyruvate and malate) and state 3 by adding 2 mM ADP and expressed as ng Atom of O₂ consumed/mg protein/min.

**Mitochondrial Permeability Transition Pore (mPTP):** mPTP opening was assessed using calcein studies performed in freshly isolated cardiomyocytes. The rate of calcein exit through mPTP was measured by recording the fluorescence signal every 2 min using a Turner Quantech Digital Filter Fluorometer with excitation filter NB490 and Emission filter SC515. The rate of calcein exit was calculated as a percent change from maximal fluorescence signal.

**Measurement of Mitochondrial Membrane Potential**

The membrane-permeant JC-1 dye, used as an indicator of MITO membrane potential (Δψm) in cardiomyocytes, exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green (495ex/534em) to red (495ex/590em). Briefly, using a commercially available kit (Sigma-Aldrich, St. Louis, MO), 10 µl of 2 fold diluted gravity settled cardiomyocytes were added in 90 µl of 0.2 mg/ml JC-1 dye with and without valinomycin (final concentration 0.1 mg/ml) working solution and incubated at room temperature for 2 minutes followed by measurement of fluorescence in microplate using a Tecan Safire fluorometer. The ratio of red to green was calculated after subtracting valinomycin-sensitive fluorescence.

**ATP Synthesis and ATP/ADP Ratio:** MITO maximal rate of ATP Synthesis and the ratio ATP/ADP were determined in freshly isolated cardiomyocytes using the ApoSENSOR™
ADP/ATP ratio bioluminescent assay kit (BioVision, Milpitas, CA). The assay utilizes the enzyme luciferase to catalyze the formation of light from ATP and luciferin. The level of ADP was measured by its conversion to ATP that is subsequently detected using the same reaction. The maximal rate of ATP synthesis was expressed in relative light units (RLU)/mg protein.

**Determination of Mitochondrial Complex I and Complex IV Activities**

**Complex I (NADH: ubiquinone oxidoreductase) Activity:** The complex I activity was measured in frozen MITO fractions (at -80°C) isolated from LV tissue. Approximately, 50 µg MITO fraction was treated with 1% cholate at 4°C for 10 minutes to solubilize the macro complex I. The activity of solubilized MITO complex-I was assayed spectrophotometrically in MITO membrane fractions isolated from LV tissue as previously described (8) by following the oxidation of NADH (0.25 mM) at 340 nm at 30°C in an assay buffer containing 62.5 μM ubiquinone, 0.25% BSA, antimycin A (2 μg/ml), and mitochondria in the absence and presence of rotenone (10 μg/ml). Considering molecular absorptivity of NADH as 6.22, Complex-I activity was calculated as the rotenone-sensitive NADH:ubiquinone oxidoreductase activity and expressed as nmoles/min/mg protein.

**Complex-IV (Cytochrome c Oxidase) Activity:** The activity of MITO complex-IV was determined polarographically as previously described (10) in MITO membrane fractions isolated from LV tissue. Briefly, using Strathkelvin resirometer, approximately 5 µg MITO protein extract (obtained after treating approximately 50 µg frozen MITO fraction
in 1% sodium deoxycholate in a 100 µl volume by incubating in ice bath for 10 minutes) was added to oxidize reduced 40 µM cytochrome c containing 1 mM N'N'N'N' tetramethyl phenylene diamine and 10 mM ascorbate, with and without 2 mM sodium azide. Sodium-azide-sensitive oxidation rate of cytochrome c was calculated and expressed as nmoles molecular O₂/min/mg protein.

**Western Blotting**

Western blotting was used to quantify changes in LV tissue levels of proteins involved in specific MITO functions/dynamics and signaling. Equal amounts of LV protein lysate was loaded for each of the 3 study groups for every protein measured. After separating proteins on 4%-20% SDS-PAGE and transferring on PVDF membrane, blots were treated with specific primary antibodies followed by the corresponding secondary antibody coupled with horse raddish peroxidase. The bands on PVDF membrane were developed by Chemiluminescence and band intensity was quantified using a BioRad Model GS-670 imaging densitometer and expressed as densitometric units (du). The following protein groups were evaluated:

**Regulators of Mitochondrial Biogenesis and Respiration:** Antibodies for endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were obtained from Life Span Biosciences, Seattle, WA. The antibody for MITO biogenesis regulating transcriptional factor peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) was purchased from Life Span Biosciences, Seattle, WA. Cardiolipin (CL) in LV myocardium was also quantified on a per milligram of mitochondrial protein. Briefly,
mitochondria protein was isolated from LV tissue and quantified as mitochondria protein/mg of noncollagen protein. Cardiolipin/mg of noncollagen protein in LV tissue was then normalized to mitochondria protein/mg noncollagen protein in LV tissue.

**Regulators of Mitochondria-Based Programmed Cell Death or Apoptosis:** Protein levels of cytosolic cytochrome c was determined in an SDS extract of 10,000 g supernatant of LV extract. Specific antibody for cytochrome c was purchased from Santa Cruz, Dallas, TX.

Specific antibody for active caspase 3 was obtained from Sigma-Aldrich, St. Louis, MO.

**Sarcoplasmic Reticulum Calcium ATPase:** Protein levels of sarcoplasmic reticulum (SR) Ca$^{2+}$-ATPase (SERCA-2a) were measured in LV homogenate.

**Results**

None of the study dogs developed acute decompensation or died during the study and none developed ventricular or atrial arrhythmias. There were no significant differences in serum electrolytes (Na+, K+, Creatinine, Glucose, chloride, and blood urea nitrogen) in either study group between samples obtained at baseline, pre-treatment and post treatment time points. All electrolytes at all time points were within normal limits for all dogs.
References


